

Exhibit 6

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## Original investigations

G. Dudin, E. W. Steegmayer, P. Vogt, H. Schnitzer, E. Diaz, K. E. Howell, T. Cremer, C. Cremer: Sorting of chromosomes by magnetic separation 111

T. Lukusa, P. Vercauteren, H. Van den Berghe, J. J. Cassiman: SCE variability in lymphocytes and fibroblasts. A controlled study 117

E. Takahashi, Y. Kaneko, T. Ishihara, M. Minamihisamatsu, M. Murata, T. Hori: A new rare distamycin A-inducible fragile site, fra(11)(p15.1), found in two acute nonlymphocytic leukemia (ANLL) patients with t(7;11)(p15-p13;p15) 124

L. Zahed, M. Murer-Orlando, M. Bobrow: Cell cycle studies in chorionic villi 127

E. Gebhart, R. Bauer, U. Raub, M. Schinzel, K. W. Ruprecht, J. B. Jonas: Spontaneous and induced chromosomal instability in Werner syndrome 135

P. J. Howard, D. Clark, J. Déarlove: Retinal/macular pigmentation in conjunction with ring 14 chromosome 140

H. Youssoufian, C. K. Kasper, D. G. Phillips, H. H. Kazazian, Jr., S. E. Antonarakis: Restriction endonuclease mapping of six novel deletions of the factor VIII gene in hemophilia A 143

F. Bernardi, G. Marchetti, S. Volinia, P. Patracchini, A. Casonato, A. Girolami, F. Conconi: A frequent factor XII gene mutation in Hageman trait 149

A. P. Read, R. C. Mountford, S. M. Forrest, S. J. Kenwrick, K. E. Davies, R. Harris: Patterns of exon deletions in Duchenne and Becker muscular dystrophy 152

R. Fodde, M. Losekoot, M. H. van den Broek, M. Oldenburg, N. Rashida, A. Schreuder, J. T. Wijnen, P. C. Giordano, N. V. S. Nayudu, P. Meera Khan, L. F. Bernini: Prevalence and molecular heterogeneity of  $\alpha$ -thalassemia in two tribal populations from Andhra Pradesh, India 157

A. H. van der Hout, A. Y. van der Veen, J. A. Aten, C. H. C. M. Buys: Localization of DNA probes with tight linkage to the cystic fibrosis locus by in situ hybridization using fibroblasts with a 7q22 deletion 161

H. H. Stassen, D. T. Lykken, P. Propping, G. Bomben: Genetic determination of the human EEG. Survey of recent results on twins reared together and apart 165

A. Hanauer, Y. Alembik, B. Arveiler, L. Formiga, S. Gilgenkrantz, J. L. Mandel: Genetic mapping of anhidrotic ectodermal dysplasia: DXS159, a closely linked proximal marker 177

J. C. Pronk, R. R. Frants, B. Crusius, A. W. Eriksson, F. de Wolf, C. A. B. Boucher, M. Bakker, J. Goudsmit: No predictive value of GC phenotypes for HIV infection and progression to AIDS 181

C. B. Eap, C. Cuendet, P. Baumann: Orosomucoid ( $\alpha$ -1 acid glycoprotein) phenotyping by use of immobilized pH gradients with 8 M urea and immunoblotting. A new variant encountered in a population study 183

## Short communications

M.-G. Mattei, M. Petkovich, J.-F. Mattei, N. Brand, P. Chambon: Mapping of the human *hap* retinoic acid receptor to the q21 band of chromosome 17 186

M.-G. Mattei, H. de Thé, J.-F. Mattei, A. Marchio, P. Tiollais, A. Dejean: Assignment of the human *hap* retinoic acid receptor RAR $\beta$  gene to the p24 band of chromosome 3 189

B. Wirth, F. H. Herrmann, M. Neugebauer, E. F. Gillard, K. Wulff, C. Stein, K. v. Figura, M. A. Ferguson-Smith, A. Gal: Linkage analysis in X-linked ichthyosis (steroid sulfatase deficiency) 191

A.-M. Rekilä, M.-L. Väisänen, M. Kähkönen, J. Leisti, R. Winqvist: A new RFLP with *StuI* and probe cX55.7 (DXS105) and its usefulness in carrier analysis of fragile X syndrome 193

## Clinical case reports

I. Pinel, A. Diaz de Bustamante, M. Urioste, V. Felix, A. Ureta, M. L. Martinez-Frías: An unusual variant of chromosome 16. Two new cases 194

I. Šubrt, K. Štirská: Familial translocation t(17;22), including the segregation in five consecutive abortions 195

J. L. Tolmie, E. Boyd, P. Batstone, M. E. Ferguson-Smith, L. Al Roomi, J. M. Connor: Siblings with chromosome mosaicism, microcephaly, and growth retardation: the phenotypic expression of a human mitotic mutant? 197

K. Naritomi, N. Hyakuna, Y. Suzuki, T. Oriti, K. Hirayama: Zellweger syndrome and a microdeletion of the proximal long arm of chromosome 7 201

E. D'Alessandro, C. De Mattois Vaccarella, M. L. Lo Re, F. Cappa, A. D'Alfonso, S. Discepoli, M. R. Della Penna, G. Del Porto: Pericentric inversion of chromosome 19 in three families 203

## Letter to the editors

C. Hausmann, E. Beck, G. Wolff, I. Volculescu: Deletion 11q23.3 without familial predisposition 205

## Announcements 206

Indexed in *Current Contents*

# Assignment of the human *hap* retinoic acid receptor RAR $\beta$ gene to the p24 band of chromosome 3

Marie-Geneviève Mattei<sup>1</sup>, Hugues de Thé<sup>2</sup>, Jean-François Mattei<sup>1</sup>, Agnès Marchio<sup>2</sup>, Pierre Tiollais<sup>2</sup>, and Anne Dejean<sup>2</sup>

<sup>1</sup> INSERM and Centre de Génétique Médicale, Hôpital d'Enfant, La Timone, F-13385 Marseille Cédex 5, France

<sup>2</sup> INSERM, U.A. 271 CNRS, Unité de Recombinaison et Expression Génétique, Institut Pasteur, F-75724 Paris Cédex 15, France

**Summary.** The human *hap* retinoic acid receptor RAR $\beta$  has been localized by in situ hybridization to the p24 band of chromosome 3.

$3.9 \times 10^7$  dpm/ $\mu$ g. The radiolabelled probe was hybridized to metaphase spreads at a final concentration of 500 ng/ml of hybridization solution as previously described (Mattei et al. 1985).

## Introduction

A new gene, named *hap* for hepatoma, has been isolated by characterizing the integration site for hepatitis B virus in a human hepatocellular carcinoma (Dejean et al. 1986). The analysis of the nucleotide sequence of the corresponding cDNA clone clearly identified this gene as a new member of the nuclear receptor multigene family (de Thé et al. 1987). The *hap* product exhibited notably a strong homology with the human retinoic acid receptor (subsequently termed RAR $\alpha$ ) (de Thé et al. 1987; Giguère et al. 1987; Petkovitch et al. 1987). We have recently demonstrated that *hap* encodes a second retinoic acid receptor, designated RAR $\beta$  (N. Brand, M. Petkovitch, A. Krust, P. Chambon, H. de Thé, A. Marchio, P. Tiollais, A. Dejean — unpublished work). Using a *hap* genomic single-copy DNA probe, we report here that the RAR $\beta$ /*hap* gene maps to chromosome 3 p24, close to the region where the thyroid hormone receptor TR $\beta$  has been located.

## Materials and methods

### Chromosome spread preparation

In situ hybridization was carried out on chromosome preparations obtained from human lymphocytes that had been phytohemagglutinin-stimulated for 72 h. 5-Bromodeoxyuridine was added for the final 7 h of culture (60  $\mu$ g/ml of medium), to ensure a post-hybridization chromosomal banding of good quality. Slides were treated with RNase and denatured prior to hybridization in 70% (vol/vol) deionized formamide, 2  $\times$  SSC (0.3 M NaCl, 30 mM sodium citrate) at 70°C.

### Probe preparation and in situ hybridization

The genomic single-copy DNA probe referred to as RT (Dejean et al. 1986) containing an insert of  $\approx$  3,500 bp in pBR327 was titrimetrically labelled by nick-translation to a specific activity of

### Autoradiography, staining and banding

After coating with nuclear track emulsion (Kodak NTB<sub>2</sub>), the slides were exposed for 8 days at 4°C, then developed. To avoid any slipping of silver grains during the banding procedure, chromosome spreads were first stained with buffered giemsa solution and metaphases photographed. R-banding was then performed by the fluorochrome-photolysis-giemsa (FPG) method and metaphases re-photographed before analysis.

## Results and discussion

In the 100 metaphases examined after in situ hybridization, there were 191 silver grains associated with chromosomes and 57 of these (29.8%) were located on chromosome 3 (Fig. 1). The distribution of grains on this chromosome was not random: 75% of them mapped to the p22–p24 region of the chromosome 3 short arm with a maximum in the 3p24 band (Fig. 2). These data strongly suggest that the retinoic acid receptor RAR $\beta$  is located on the p24 band of chromosome 3. The retinoic acid receptor RAR $\alpha$  has been recently located to the q21 band of chromosome 17 (Mattei et al. 1988). It is interesting to note that the RAR $\alpha$  and RAR $\beta$  are more homologous to the two closely related thyroid hormone receptors TR $\alpha$  and TR $\beta$  than to any other members of the nuclear receptor family (de Thé et al. 1987; Giguère et al. 1987; Petkovitch et al. 1987). The thyroid hormone receptor TR $\beta$  maps to chromosome 3p21.33–22 (Drabkin et al. 1987), the thyroid hormone receptor TR $\alpha$  (also termed *erba1*) maps most probably to chromosome 17q11.2–q12 (Robertson 1987) while two other *c-erba*-related genes, *erba2* and *erba2-like*, have been mapped respectively to chromosome 17q21.3 and 17q25 (Gosden et al. 1986).

Analysis of the gene family encoding the nuclear receptors has shown that they can be roughly divided into two groups: the steroid receptors, which have different chromosomal localizations (Mattei et al. 1988), and the non-steroid receptors, which appear to be located on either chromosome 3 or 17. This observation suggests that the genes encoding the

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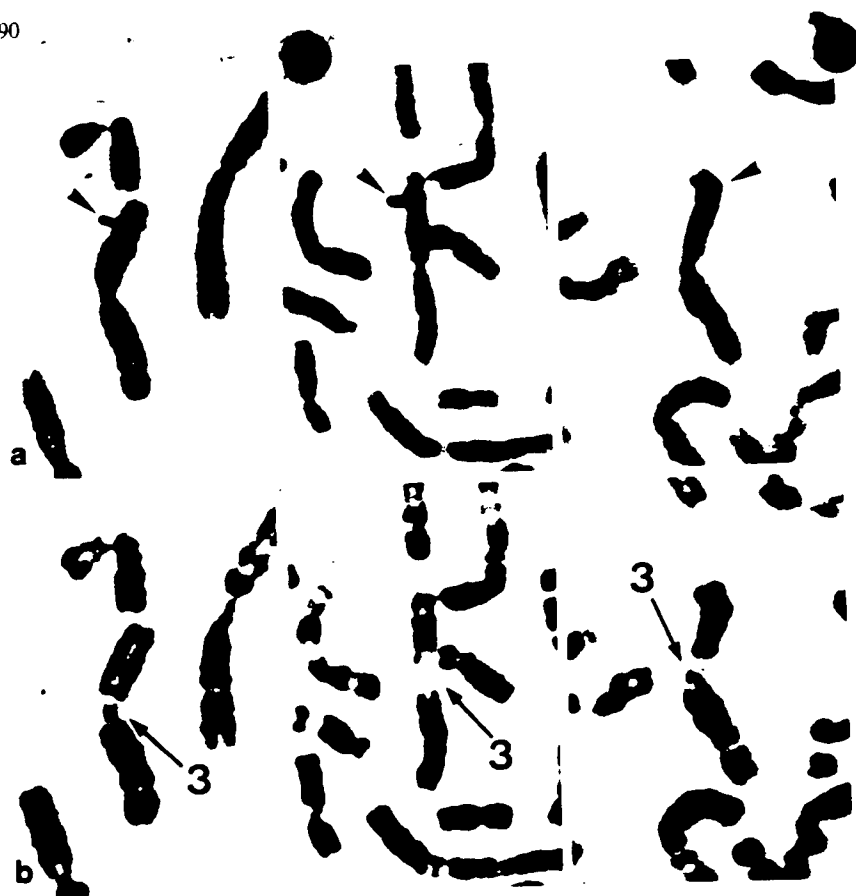


Fig. 1a, b. Three partial human metaphases showing the specific site of hybridization to chromosome 3. a Arrowheads indicate silver grains on Giemsa-stained chromosomes after autoradiography. b The same chromosomes with silver grains were subsequently identified by R-banding (FPG technique)

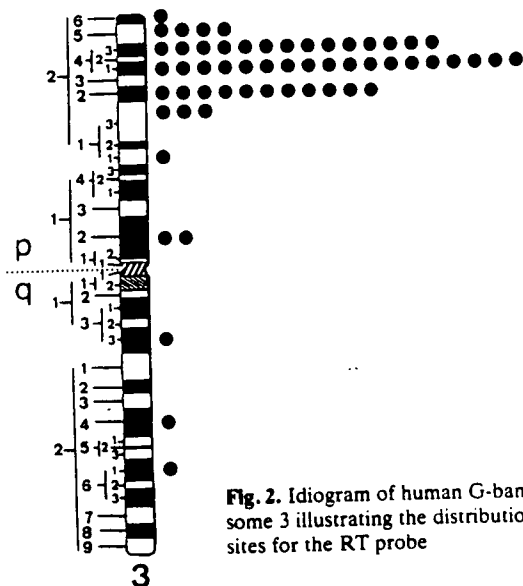


Fig. 2. Idiogram of human G-banded chromosome 3 illustrating the distribution of labeled sites for the RT probe

thyroid hormone and retinoic acid receptors have evolved by duplication of an ancestral gene, which itself diverged earlier in evolution from the steroid hormone receptor progenitor.

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